

Bacterial and fungal colonization on metallic and ceramic orthodontic brackets: A scanning electronic microscopy study

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Abstract - *Universally, aesthetic smiles are common desires among people and can be achieved through orthodontic braces. In the human mouth, as well as on the surface of the teeth, a plentiful microbial community coexists, characterizing the biofilm. The aim of this study was to verify the pattern of bacterial (*Streptococcus mutans* and *Lactobacillus* spp.) and fungal (*Candida albicans*) colonization on metallic and ceramic brackets (3M Unitek). Partial fixed appliance were installed in 18 patients and two plaque collections were made: first - directly from dental surface; second - 21 days after bonding procedures, from brackets surfaces. Specific laboratory tests were carried out and for the fulfillment of the macroscopic reading, plates that presented from 30 to 300 colonies were selected. Scanning electron microscopy (SEM) examinations were performed on the surface of the brackets after 28 days of the experiment. *S. mutans* were the prevalent microorganisms, followed by *Lactobacillus* spp. and *C. albicans*. No obvious pattern of microorganism colonization favoring one bracket material over the other was found. Positive correlation was observed on the presence of *Candida albicans* between the initial condition and after braces insertion in the mouth. SEM showed heterogeneous distribution forms of cocci, bacilli, yeasts and filamentous fungi in the three areas delimited for visualization on bracket surface. All species investigated were present on the braces and we concluded that there is no difference when comparing ceramic and metallic brackets. Microorganisms did not show sites of preference in colonization, however, the slot areas presented greater accumulation.*

I. INTRODUCTION

Globally, orthodontic treatment of malocclusions is based on mechanical energy generated by fixed

orthodontic appliance forces[1], however the orthodontic devices, such as brackets, may provide additional retentive surfaces for oral microorganism[2]. The diversity can

promote alterations in oral environment, greater adherence of microorganisms and development of biofilm[3, 4].

The buccal microbial community is a mixture of different microorganisms[5, 6] and some of them are associated with enamel demineralization such as *Streptococcus mutans*[7, 8] and *Lactobacillus spp.*[9, 10]. There is also a direct relationship between gingival inflammation[11], dental plaque[12, 13] and the frequency of *Candida* species can also be increased by the presence of these devices[14, 15]. *Candida* species are present in about 50-60% of global population[16] being linked to an infection called candidiasis[17, 18].

Orthodontic treatment has been increasingly requested by young and adult patients mainly due to the constant increase of aesthetic requirements and the search for a pleasant appearance[19]. Treatments with discrete bracket and aesthetic devices are highly requested by patients nowadays[20].

The rough surface of the brackets provides a favorable ecologic niche for the adherence of microorganisms living in a microbial biofilm community[21] presenting continuous development[22, 23]. Several studies have analyzed the bacterial adhesion on different types of metal and ceramic brackets[1, 24, 25], but few studies evaluate the colonization of these devices according to the type of brackets.

The present study aimed to evaluate the presence of *Streptococcus mutans*, *Lactobacillus spp.* and *Candida albicans* of the buccal microbiota before and after the devices bonding, as well as, to analyze how the colonization of these microorganisms is distributed over aesthetic and metallic brackets in different zones of these accessories by scanning electron microscopy (SEM).

II. METHODS

2.1 Sample collection

Twenty volunteers were randomly selected and all of them had complete permanent dentition. Exclusion criteria included orthodontic treatment, carious lesions, periodontal complications and antimicrobial use in the last 3 months before the clinical study. The procedures for conducting the research were approved the Human Ethics Committee of the University Hospital Clementino Fraga Filho of the Federal University of Rio de Janeiro – Brazil, by the number 2.796.767.

The subjects received oral hygiene instructions, with the intention of standardizing tooth brushing during the study. The modified Bass technique was taught, and also, an oral hygiene kit that included a toothbrush (Oral B/Proctor & Gamble) and toothpaste (Colgate-Palmolive)

was given. There was a loss of the sample and the survey ended with 18 members.

2.2 First biofilm collection

One week after the oral hygiene instructions, the first biofilm samples were collected. This step was performed before bonding in order to identify which microorganisms were presented on the teeth in this first moment and to determine the biofilm profile of each patient. The patients were instructed to not eat food and to not brush their teeth for a minimum of 12 hours before the collection of the dental biofilm. Plaque was collected with individual sterile curette, obtained from the upper and lower canines and first premolars from the supragingival areas: cervical-buccal, mesial-interproximal and distal-interproximal surfaces[4].

Three groups of microorganisms were investigated: *S. mutans*, *Lactobacillus spp* and *C. albicans*. The material collected from the dental plaque on the enamel surface was placed in Eppendorf plastic tubes, disposable and sterilized with capacity of 1.5 ml. All the empty tubes were identified and weighted on a precise electronic micro scale before and after being used in laboratory procedures in order to obtain and quantify the actual amount of plaque. The proportion in the initial dilution was standardized and homogenized on a mechanical vortex, using for each 1 mg of plaque collected 1 ml of sterile reducing saline solution composed (0.85% sodium chloride supplemented with 1% sodium thioglycolate). In this step, subsequently, 0.1 ml aliquot was removed from the Eppendorf tube and placed in a test tube containing 0.9 ml of the same saline dose being homogenized one more time. The serial decimal dilutions ranged from 10^{-1} to 10^{-4} , always carrying 0.1 ml of the previous dilution. This dilution led to a decrease in the number of colonies facilitating counting by visual inspection. After that, 0.1 ml aliquots of each dilution were sown in Petri dishes containing specific culture substances for each type of microorganism.

2.3 *Streptococcus mutans*, *Lactobacillus spp* and *Candida albicans*

To analyze *S. mutans* it was used a selective Mitis Salivarius agar modified by the addition of 20% sucrose and 0.2 µl bacitracin per ml. The plates were incubated under anaerobic conditions at 37 °C for 48 hours. The *Lactobacillus spp* were assessed by cultivation on Rogosa agar at 37 °C for 72 hours. Finally, to analyze *C. albicans*, it was used a selective CHROMagar Candida medium at 37°C for 48 hours. For all the experiments were selected plaques that presented from 30 to 300 macroscopically visible colonies. Then, the colonies were counted and the inoculated amount was converted by the 10^{-1} , 10^{-2} , 10^{-3} and

10^{-4} dilution factor[4, 26].

2.4 Bonding brackets

The study proceeded with the bonding of eight brackets in each patient with ones being positioned on upper canine and first premolar, and lower canine and first premolar. Two types of Brackets were used: metal (3M UNITEK) and ceramic Clarity brackets (3M UNITEK) both with a 0.022 slot for edgewise-arch technique and prescription for canines and bicuspid tooth. The design for

each patient was composed as follows: tooth 13/ceramic and tooth 14/metallic; tooth 23/metallic and tooth 24/ceramic; tooth 33/ceramic and tooth 34/metallic; tooth 43/metallic and tooth 44/ceramic (Figure 1).

The slot of these brackets were filled with passive 0.019" x 0.025" rectangular wire segments, tied with 0.010" metallic wire simulating a real installation in the mouth, which are considered parts of conventional brackets because of their function on the appliance[1].

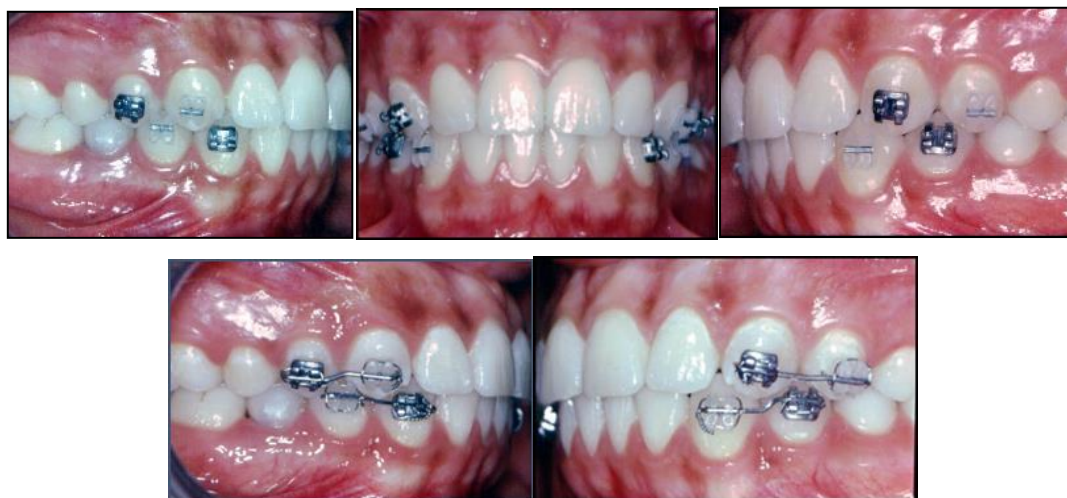


Fig. 1: Final composition of the appliance design with metallic and ceramic brackets.

2.5 Second biofilm collection

This collection was done 21 days after bonding the brackets and was obtained from the surfaces of halets, slots and cervical regions. The passive 0.019" x 0.025" rectangular wire segments that were inside slot were removed from all brackets leaving the slots free. The material collected was placed in 1.5 mL, sterile, plastic, disposable Eppendorf tubes (Axygen, Union City). These tubes were identified for each patient, tooth and type of bracket, being weighed by precise electronic balance (model BG200) following the same criteria adopted in the first biofilm collection to identify *S. mutans*, *Lactobacillus* spp. and *C. albicans*.

2.6 Debonding brackets and Scanning Electron Microscopy

After the procedures performed in the first and second microbial collection, the brackets remained positioned on the teeth in order to visualize in SEM[27] the

structural arrangement of the biofilm on the metallic and ceramics surfaces. In addition, the patients were instructed on the need for the appliance to remain in the oral cavity for more another 7 days. Otherwise, if these brackets were removed in this same session (second microbial collection), the microscopic visualization of the colonized surface would be altered, given the scraping performed.

So, the patients were informed to suspend tooth brushing only on the 28th day in order not to damage the colonies located on the brackets. To perform the removal of these brackets to be prepared for SEM were used an orthodontic pliers and the handling of the study bodies followed the steps below:

a) the brackets were packed in an acrylic plate with 24 numbered wells, identifying the tooth and the patient to which each bracket belonged, and subjected to fixation through the gradual series of alcohols concentration from

50% to 70%, 75%, 90% and 100%. In each concentration, the accessories remained submerged for 10 minutes;

b) any water residue that may have been in this microbial material was eliminated through Dehydration at a Critical Point with the aid of the “Critical Point Dryer” model CPD 030; Bal-Tec AG, Balzers. In a chamber, the combination of temperature variation ranging from 10°C to 38°C, plus gas exchange (CO₂), (liquid CO₂ + alcohol) and pressure increase, oscillating between 80 to 90 atm, promoted by this device, created if a completely dry medium[4];

c) after the pieces were dehydrated, they were glued with silver-based adhesive to the upper surface of the “stubs” and then subjected to metallization with gold coating on the Balzer Union FL 9496 - BalTec AG apparatus;

d) once metallized, the pieces were placed in an acrylic well plate and then examined in a Scanning Electron Microscope JEOL-JSM 5310, with a magnification of 35x, 1,000x, 2,000x, 5,000x and 7,500x in order to verify the arrangement of microorganisms in the composition of the colonies.

Three areas were delimited for visualization on the surfaces of each accessory: 1) central area of the occlusal mesial wing; 2) central area of the mesial slot on the metal brackets and central zone of the slot on ceramic brackets; 3) central zone of the distal cervical wing.

2.7 Statistical analysis

Descriptive statistics procedures were used to express the results as median and interquartile range (IQR). The normality of the data was tested using the Shapiro-Wilk test. The comparisons between the microorganisms were

made using the Kruskal-Wallis test, with the comparisons between pairs using the Mann-Whitney test.

The Mann-Whitney test was also used to test the differences between the types of brackets. Correlations of Pearson and Spearman were used to test the associations between the different microorganisms counts in the baseline: before the brackets bonding and after 21 days of the procedure.

The significance level adopted was 5% ($\alpha = 0.05$) and the analyzes were performed in IBM SPSS Statistics for Windows (IBM SPSS, 21.0, 2012, IBM Corp).

III. RESULTS

The purpose of the present study was to compare the bacterial and fungal present on orthodontic appliances in order to clarify which bracket type has a higher plaque retaining capacity and to determine the levels of *S. mutans* and *Lactobacillus* spp. and *C. albicans* on both types of brackets: metallic and ceramic.

The first biofilm collection and quantification of colony forming units (CFU) from microorganisms located directly on tooth surface, revealed the following means values of bacterial and fungal: *Streptococcus mutans* 13.29, *Lactobacillus* spp. 4.17 and *Candida albicans* 3.26.

Our study focused on microbial colonization, as well as the pattern in the oral microbiota before/after orthodontic appliances installation and furthermore made it possible to obtain more illustrative data about the microbial population directly allocated on the surface of these accessories (Tables 1 and 2).

Table 1. Correlations between the counts of the three different microorganisms obtained from dental enamel before brackets bonding and on the patients brackets surface after 21 days*.

Microorganism	Bracket Types	
	Metallic	Ceramic
<i>Streptococcus mutans</i>	$r_{\text{Pearson}} = 0,18$ (p = 0,476)	$r_{\text{Pearson}} = 0,18$ (p = 0,476)
<i>Lactobacillus</i> spp.	$r_{\text{Spearman}} = 0,03$ (p = 0,904)	$r_{\text{Spearman}} = 0,24$ (p = 0,336)
<i>Candida albicans</i>	$r_{\text{Spearman}} = 0,62$ (p = 0,006)	$r_{\text{Spearman}} = 0,52$ (p = 0,026)

*No significance was found for *Streptococcus mutans* and *Lactobacillus* spp., however, positive and moderate correlation was observed on the presence of *Candida albicans* between the initial condition and in the final condition, after the insertion of fixed orthodontic appliances in the oral cavity.

Table 2. Colony forming units (CFU) of the different microorganisms obtained from the patients brackets surface after 21 days of the brackets bonding.

Microorganism	CFU/mL in different types of brackets		*p-value
	Metalic	Ceramic	
<i>Streptococcus mutans</i>	13,06 ± 1,58 ^a	12,32 ± 2,93 ^a	0,393
<i>Lactobacillus</i> spp.	7,88 ± 3,33 ^b	7,23 ± 3,88 ^b	0,862
<i>Candida albicans</i>	2,30 ± 4,22 ^c	0,00 ± 4,63 ^c	0,342
†p-value	< 0,001	< 0,001	

The results are expressed as median ± interquartile range.

* Mann-Whitney test; † Kruskal-Wallis test: ^{a,b,c} distinct letters (column) indicate statistical difference between the microorganisms by the Mann-Whitney test.

IV. DISCUSSION

In the current study comprising fixed appliances, the levels of bacterial and fungal species on both metallic and ceramic orthodontic brackets were examined by laboratorial tests and scanning electronic microscopy. Our data suggest that differences in the bacterial composition of dental plaque formed on each bracket type exist, however, the composition is, for the most part, very similar between the two bracket types. The differences detected certainly do not favor one bracket type over another one with respect to other bacterial and fungal accumulation present in the biofilm. Even though the statistical analyzes did not reveal great significance, the study proved to be quite relevant because when we have to choose between aesthetic and conventional brackets, the bacterial colonization requirement will not prevail over this decision.

In this study when the three species studied were analyzed, it was observed that the *S. mutans* was present, in a balance way, on the dental surface of all the individuals, before bonding procedure, in contrast to the numbers of *Lactobacillus* spp and *Candida albicans* (Table 1). Differences were noted in relation to *Lactobacillus* spp. and *C. albicans* whose presence of one species is marked by the absence of another. This finding was observed in our study in which the increase in the CFU rate of *Lactobacillus* spp. was accompanied by the decrease in *C. albicans* (Table 1). Some researchers studying biofilm[28] found that the lactobacilli inhibit early stages of *C. albicans* biofilm development by reducing its growth, cell adhesion, filamentation (yeast-to-hyphae differentiation) and biofilm formation. The inhibitory effects of the probiotic *Lactobacillus* on *C. albicans* entail both cell-cell

interactions and secretion of metabolites that may impact on pathogenic attributes associated with *Candida albicans* colonization on host surfaces and yeast filamentation. This clarified the mechanism of how *Lactobacillus* species may antagonize *C. albicans* host colonization[28].

Some studies providing an investigation about orthodontic appliances among children on salivary levels of *S. mutans*, *Lactobacillus* spp. and *C. albicans* for six month follow-up[29], revealed that *S. mutans* and *Lactobacillus* spp. counts increased significantly 6 months after the insertion of orthodontic appliances in the oral cavity, moreover a significant increase in *C. albicans* counts was noted after 3 months compared with baseline. In our study conducted in 21 days using fixed appliance, we could realize that the values for microorganisms (*S. Mutans*, *Lactobacillus* spp. and *C. albicans*) also increased after appliance installation, however, statistical significance was noted only when numbers of *C. albicans* was compared.

In our research the microbial colonization on metallic brackets showed a slight predominance of *S. mutans*, with no obvious significance when compared to the ceramics (Table 2). This situation is in agreement with the results found by some researchers[30] that studied the profile of bacteria, whose material was collected directly from the surface of metal and ceramic brackets. Comparing the two types of accessories, the behavior of microorganisms in our research seem to be in agreement with the results found by these authors, who described that there are no significant differences between the colonization of metal and ceramic brackets, with a predominance of *S. mutans* and *Lactobacillus* spp., in this decreasing order.

On the other hand, in a comparative study[31] of long-term biofilm formation on metal and ceramic brackets was found some kind of relevance in results indicating that ceramic brackets exhibit less long-term biofilm accumulation than metal brackets.

In the current evaluation, the colonization pattern evidenced by *S. mutans*, in relation to ceramic brackets, showed the lowest mean of variation when comparing the three types of surfaces: dental, metallic and ceramic. These data seem to be in disagreement with the results shown by previous studies[32], who, evaluating the adhesion and affinity of *Streptococcus mutans* for metal, plastic and ceramic orthodontic brackets, *in vitro*, verified the higher affinity of these microorganisms for the surface of ceramic brackets than by the surface of metal or plastic brackets.

In the present work, it was possible to verify that the correlations between the counts of the different microorganisms obtained from dental enamel, in the beginning, and from the brackets surface of the patients, after 21 days of bonding accessories, were subtle. Positive and moderate correlations were observed between the presence of *Candida albicans* in the initial condition and in the final condition. No correlation was found for *S. mutans* and *Lactobacillus* spp. The numbers reveal a similar situation to the studies outlined by authors[3] assessing levels of microorganisms in patients before, during, and after orthodontic treatment.

Numbers of a results from an *in vitro* study[32] demonstrated that adhesion of *S. mutans* was weaker on metallic than on plastic and ceramic brackets, indicating that metallic brackets had a lower potential for bacterial accumulation than plastic and ceramic brackets. However, despite these differences *in vitro* adhesion, the present study, conducted by us *in vivo*, suggests that this may have effect on the microbial populations that colonize orthodontic brackets *in vivo*. A positive correlation[33] was found between the surface roughness and biofilm adhesion *in vivo* than *in vitro* experiment when *Streptococcus mutans* and *C. albicans* were studied.

An *in vitro* study[1] revealed the influence of four different types of fixed orthodontic appliances (two metal and two ceramic) on the growth and adherence of microorganisms, including *S. mutans* and *Candida albicans*. Those authors showed significant differences between the different appliances providing a vision on the adhesion of bacteria and fungi on the orthodontic accessories. According to those authors[1], yeasts like *C. albicans* species, are the most frequently found microorganism in infections of buccal mucosa and showed more adherence than *S. mutans* with all types of appliances used, and its adherence to metallic brackets is higher than

those with esthetic appearance. These findings contrast completely with the numbers obtained by us in our current research.

Studying the influence of different orthodontic brackets on adherence of microorganism *in vitro*, authors[34] using three types of brackets (metallic, ceramic and composite) concluded that the adherence of *S. mutans* was not modified by the differences among brackets, however, the adherence of *C. albicans* was increased by the composite bracket. The use of metallic brackets seem to decrease yeast adherence and the number of colony forming units (CFU), while composite aesthetic brackets facilitated it. These values are in agreement with the results achieved in our study with respect to *S. mutans* but not with respect to *C. albicans*. Verifying by SEM, these authors[34] demonstrated that the adherence of *S. mutans* plus *C. albicans* together varied according to the bracket materials: composite > ceramic > metallic. In our research, the SEM observation highlighted heterogeneous distribution of microorganisms on the two types of brackets.

Assessment conducted by authors comparing *Candida* spp profile in patients with fixed and removable orthodontic appliances therapy[35] concluded that the first ones promotes an increase in levels of the yeasts in particularly non-albicans *Candida* species. These data are in agreement with the numbers found in the present study (Table 2), which show increasing values for *Candida albicans*, after assembling the apparatus, for the two types of brackets studied.

Authors evaluating the effects of orthodontic appliances on *Candida* in the human mouth[18] demonstrated that the most common *Candida* species isolated in the orthodontic patients was *C. albicans* and that there seems to be a direct relationship between the presence of: appliances, *Candida* and low salivary pH levels. No healthy patients developed *Candida* infection from the orthodontic appliances. In our study no healthy patients in the sample developed complications due to *Candida*. The differences in the bacterial and fungal adhesion amount can be explained by the difference in the surface characteristics of each material, including the surface roughness: stainless steel and monocrystalline ceramic. This interaction between microorganism and hard surface must be taken into account. In our research, worth mentioning that an important factor that may explain the differences with the previous studies is the combination of bracket and arch wire together to simulate the fixed orthodontic appliance inside the patient mouth, which may provide more retentive surface for the formation of dental plaque creating a real situation.

In our study *S. mutans* was the most prevalent microorganism, followed by *Lactobacillus* spp. and *C.*

albicans. No statistical differences were found considering the counts of microorganisms and the type of bracket studied.

4.1 Scanning Electron Microscopy

Orthodontic treatment can be performed in different ways with many types of appliances that may contribute to new stagnant areas susceptible for colonization and retention of species. Studies showing the magnification of microbial niches, as well as the biofilm located on the brackets, are still needed. The scanning electron microscopy (SEM) examination on the surface of the metal and ceramic brackets showed a heterogeneous distribution of forms suggestive of bacterial *cocci* and *bacilli*, as also yeast and filamentous fungi in the three areas delimited for visualization (Figures 2 to 10).

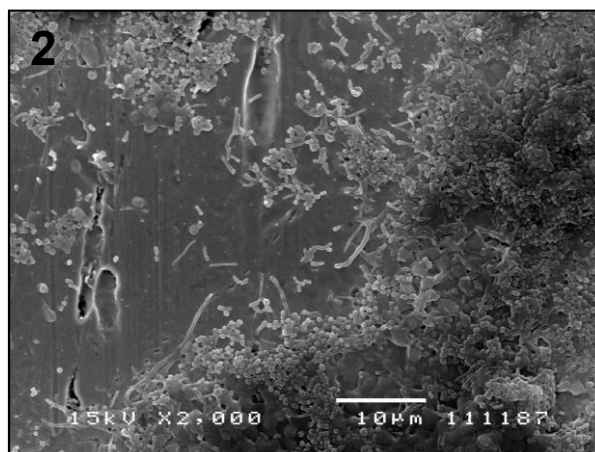
In our SEM images, the biofilm could be seen with forms suggestive of *cocci*, *bacilli* and *fungi* forms. These microorganisms were present in the three areas delimited for investigation and subtle differences in the distribution of microflora and colonization could be observed. In both types of brackets, *S. mutans* was the most prevalent microorganisms, followed by *Lactobacillus* spp. and *Candida albicans*.

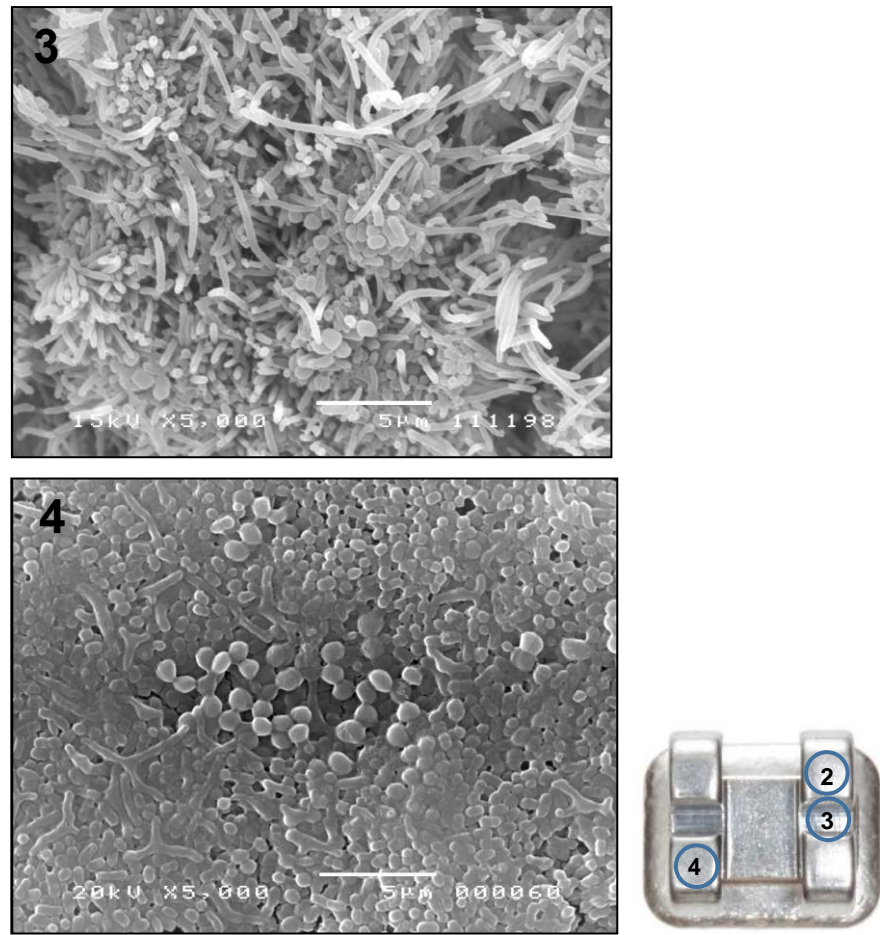
Ultrastructurally, our images from metallic and

ceramic bracket surfaces showed a colonization pattern of microorganisms with densely inhabited areas whose structures suggest *cocci*, *bacilli* and filamentous fungi forms

These data are in agreement with some work[27] that investigated plaque distribution on bonded brackets through SEM and found typical plaque morphology characterized by filamentous, rod and spheroidal groups (*fungi*, *Lactobacillus* and *Streptococcus*) showing mixed aggregation. The results evidenced by authors[4] tells us that in the distinction between the brackets, with greater or lesser formation of colonies, was observed that there was discreet colonization on the hooks of the brackets and the greatest formation of colonies occurred in the slots. In our study the slot region, in both types of brackets, shows itself as a field of greater co-agglomerations among species and it is worth mentioning that the ceramic bracket slot (clarity brack 3M) consists of a metal surface. In some images it was possible to show the exact moment of the sprouting of a fungi form suggestive of *Candida* species (Figure 11).

We also concluded and agree with many other authors[29] that long-term utilization of orthodontic appliances may have a effect on microbial flora and it is recommended that patients be recalled within short time intervals to be motivated for oral hygiene during their orthodontic therapy.



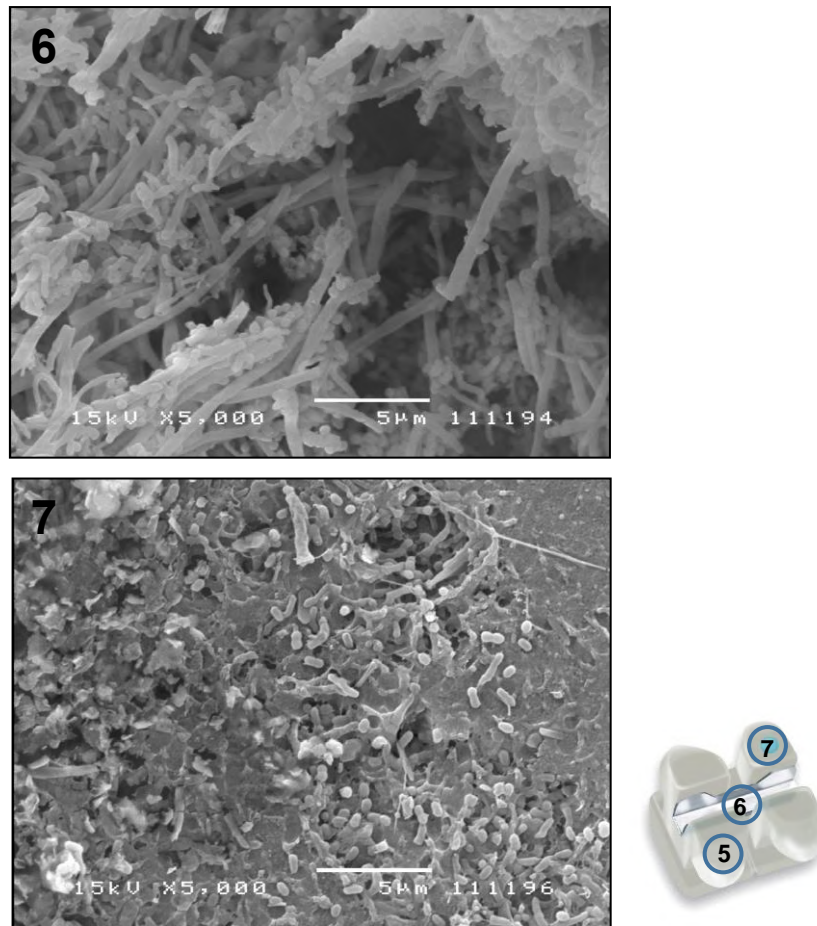


SEM images from delineated fields of investigation on metallic bracket surface showing plaque accumulation and the pattern of colonization performed by microorganisms (patient 11, tooth 43).

Fig. 2) visualization of the aggregation evidencing suggestive spherical shapes accompanied by rods; *Fig. 3)* central area of the slot showing abundant and complex biofilm composed by aggregation of bacteria in form of coccus, rods and filamentous structures suggesting fungi; *Fig.4)* metallic wing surface completely colonized by coccus in the observation field.

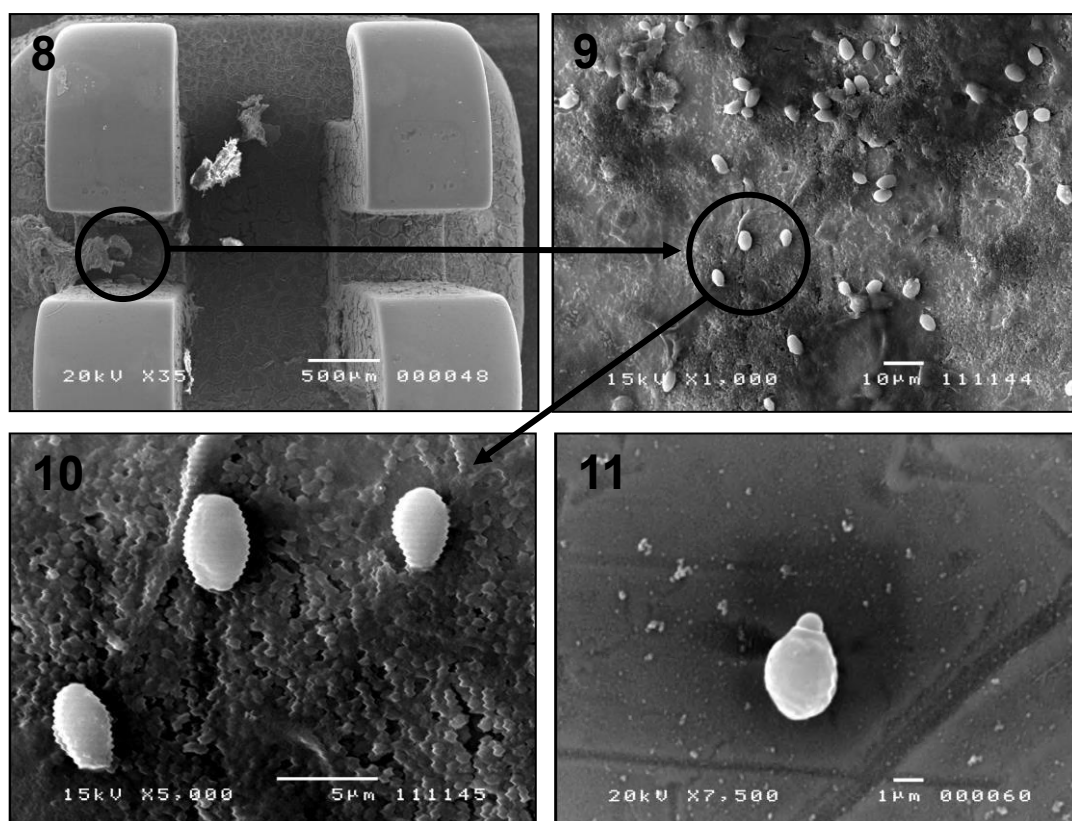
Original magnifications: *Fig. 2)* 2,000x, *Fig. 3)* 5,000x and *Fig. 4)* 5,000x





SEM images - ceramic bracket surface (patient 16, tooth 24).

Fig. 5) microbial biofilm composed by forms suggesting coccus, rods and filamentous fungi; Fig. 6) local plaque retention with aggregation of spherical microorganisms, in the form of rods and filamentous fungi (yeasts and hyphae) in the region comprised by the slot; Fig.7) superficial layer partially removed with exposure of structures suggesting bacilli and coccus. Original magnifications: Fig. 5) 5,000x, Fig. 6) 5,000x and Fig. 7) 5,000x



SEM images of the pre-outlined areas on the metallic bracket surface (patient 18, tooth 34).

Fig. 8) total taking image of the bracket with macroscopic view from plaque material; **Fig. 9)** visualization of the central zone of the "slot"; **Fig. 10)** colonization by microorganisms whose morphology suggests fungal forms (yeasts/blastospores) allocated in the biofilm surface layer. **Fig. 11)** Fungi at the time of budding reproduction – sprouting time.

Original magnifications: **Fig. 8)** 35x, **Fig. 9)** 1,000x, **Fig. 10)** 5,000x and **Fig 11)** 7.500x

V. CONCLUSION

The presente study indicates that there are no significant differences when comparing the colonization of *Streptococcus mutans*, *Lactobacillus* spp. and *Candida albicans* over metallic and ceramic brackets. Positive and moderate significance was observed in relation to the presence of *Candida albicans* between the initial condition and in the final condition, after the insertion of fixed orthodontic appliances in the oral cavity.

The SEM analyzis on the surface of metallic and ceramic brackets show that the distribution of microorganisms was marked by a decreasing scale of *Streptococcus mutans*, constituting the highest expression group, followed by *Lactobacillus* spp. and finally *Candida albicans*. These finds did not show sites of preference in colonization, however the slot areas presented greater accumulation and were colonized by microorganisms whose forms showed the coaggregation of *cocci*, *bacilli* and *fungi*.

Abbreviation Key: SEM: scanning electron microscopy, CFU: colony forming units

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